Statistical Analysis Plan

Intestinal microbiota transplant as a strategy to enhance the resilience capacity of older adults aiming to preserve muscular, cognitive, and metabolic functions in a stressful environment

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Purpose. This Statistical Analysis Plan prespecifies the efficacy, safety, and exploratory analyses for the randomized, double-blind, placebo-controlled clinical trial evaluating intestinal microbiota transplant (IMT) from young physically active donors in older adults.

1. Administrative information

This document prespecifies the statistical methods that will be used for the analysis of the randomized, double-blind, placebo-controlled trial of IMT versus placebo in older adults. It is intended to define the confirmatory and supportive analyses before database lock and before inspection of comparative outcome data.

This SAP is based on the approved protocol and subsequent amendments available as of the date listed on the cover page. Where the protocol contains broad objectives or multiple potential outcomes, this SAP establishes a formal hierarchy in order to preserve transparency, reduce analytical flexibility, and distinguish confirmatory analyses from exploratory analyses.

2. Study overview

- Study design: randomized, double-blind, placebo-controlled parallel-group clinical trial.
- Population: 80 older adults aged 65-84 years.
- Allocation: 1:1 to IMT from young physically active donors or matching placebo, using a block randomization method with concealed block sizes to ensure balanced allocation.
- Visits for repeated efficacy assessments: baseline, Week 4, Week 8, and Week 20 after IMT; cognition assessed at baseline, Week 8, and Week 20.
- Main scientific framework: muscular effects are prioritized, with cognitive and metabolic consequences evaluated as key downstream domains, and microbiome-related analyses used to explore biological mechanisms.

3. Analysis objectives

The primary objective is to evaluate whether IMT improves muscular function relative to placebo in older adults. Secondary objectives are to evaluate whether IMT influences frailty, cognition, metabolic/hepatic function, safety, and microbiome-related measures, and whether baseline perceived stress modifies treatment effects.

Additional analyses of biologically plausible associations may be conducted. Any analysis not explicitly listed as primary, key secondary, or secondary in this SAP will be considered exploratory and hypothesis-generating.

4. Endpoint hierarchy

A single primary endpoint is prespecified. A limited number of key secondary endpoints are identified to preserve interpretability and control multiplicity. Other outcomes remain prespecified secondary or exploratory outcomes.

Prespecified endpoint hierarchy

Tier	Endpoint	Primary timepoint / scale	Planned analysis
Primary	Handgrip / isometric dynamometry	Baseline to Week 20	MMRM / linear mixed-effects model
Key secondary	Combined frailty index (FI-combined)	Baseline to Week 20	MMRM / linear mixed-effects model
Key secondary	Global cognitive composite z-score	Baseline to Week 20	MMRM / linear mixed-effects model
Key secondary	Metabolic/hepatic composite	Baseline to Week 20	MMRM / linear mixed-effects model
Secondary	Other muscular, cognitive, metabolic, microbiome and safety outcomes	As specified	Family-wise or FDR-controlled analyses where applicable

5. Prespecified endpoints

5.1 Primary endpoint

The primary endpoint is the change in handgrip / isometric muscle strength measured by dynamometry from baseline to Week 20. The confirmatory estimand is the adjusted between-group difference in mean change over time, with emphasis on the contrast at Week 20.

5.2 Key secondary endpoints

1. Change in combined frailty index (FI-combined) from baseline to Week 20.
2. Change in a prespecified global cognitive composite z-score from baseline to Week 20.
3. Change in a prespecified metabolic/hepatic composite from baseline to Week 20.

The global cognitive composite will be constructed using standardized scores from cognitive tests representing attention, executive function, working memory, language and processing speed, with direction harmonized so that higher values indicate better performance. The metabolic/hepatic composite will summarize fasting glucose-insulin regulation, lipid profile, liver enzymes, and non-invasive hepatic burden markers after prespecified standardization and direction harmonization.

5.3 Secondary muscular outcomes

- Frailty phenotype category (non-frail / pre-frail / frail).

- Lean mass and fat mass by DEXA.
- Functional autonomy by GDLAM battery.
- Rectus femoris thickness and pennation angle by muscle ultrasound.
- Physical activity and sleep metrics from actigraphy.
- Blood biomarkers relevant to muscular health and inflammation, including CK, skeletal muscle troponin T, IGF-1, C-reactive protein, vitamin D, TNF-alpha, IL-6, IL-1beta, and lactate.
- PBMC-based mitochondrial respiration and autophagy-related markers.

5.4 Secondary cognitive and psychosocial outcomes

- Mini-Mental State Examination (MMSE).
- Trail Making Test A and Trail Making Test B.
- Digit span forward and backward.
- Frontal Assessment Battery (FAB).
- Verbal fluency (FAS).
- Ryff psychological well-being score.
- Subjective happiness score.
- Life satisfaction score.

5.5 Secondary metabolic and hepatic outcomes

- Fasting glucose, insulin, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, ALT, AST, and gamma-GT.
- Presence or grade of NAFLD assessed by ultrasound, if an ordinal or categorical classification is available.
- Serum MMP-9 and IL-6.
- Non-invasive hepatic fibrosis scores derived according to protocol definitions.

5.6 Safety outcomes

- Any adverse event.
- Treatment-related adverse events.
- Serious adverse events.
- Discontinuation due to adverse events.
- Solicited and unsolicited tolerability information collected during follow-up visits and scheduled contacts.

5.7 Microbiome and mechanistic outcomes

- Alpha-diversity indices and beta-diversity metrics derived from 16S or shotgun sequencing, depending on the final laboratory platform.
- Differential taxonomic and pathway abundance analyses.
- Short-chain fatty acid measurements in stool.
- Associations between microbiome-related measures and muscular, cognitive, metabolic, and stress-related outcomes.

6. Analysis populations

Analysis set	Definition	Primary use
Intent-to-treat (ITT)	All randomized participants analyzed according to assigned group, irrespective of adherence.	Primary efficacy analyses
Per-protocol (PP)	Participants without major protocol deviations and with adequate intervention exposure and outcome assessment.	Sensitivity analyses
Safety set	All participants who receive study product or placebo.	Adverse event and tolerability analyses

7. General statistical principles

- All statistical tests will be two-sided unless otherwise stated.
- The nominal type I error rate for the primary endpoint will be 0.05.
- Continuous outcomes will be summarized using mean and standard deviation or median and interquartile range, depending on distribution; categorical outcomes will be summarized using counts and percentages.
- Effect estimates will be presented with 95% confidence intervals in addition to p-values.
- Baseline comparability will be described but not formally significance-tested for the purpose of evaluating randomization success.
- Analyses will be performed primarily in the ITT population, with PP analyses treated as sensitivity analyses.
- Statistical analyses will be conducted using validated software such as R, Stata, SAS, or equivalent validated statistical platforms.

8. Covariates and baseline adjustment

The main adjusted models will include treatment group, study visit, treatment-by-visit interaction, and the baseline value of the corresponding endpoint where appropriate. Prespecified covariates for adjusted efficacy analyses will include age and sex. Education will be added for cognitive models. Baseline perceived stress score (PSS-14) will be included as a prespecified effect-modification variable in dedicated interaction models and may also be included as a covariate in supportive adjusted analyses. Additional baseline covariates may be included if clearly justified by protocol-defined measurement structure or extreme imbalance, but any such departure from the core model will be identified as supportive rather than primary.

9. Primary efficacy analysis

The primary analysis of dynamometry will use a mixed model for repeated measures (MMRM) or an equivalent linear mixed-effects model including fixed effects for treatment group, visit, and treatment-by-visit interaction, with participant as a random effect. An unstructured covariance matrix will be preferred when supported by the data; if convergence issues arise, a more parsimonious covariance

structure such as first-order autoregressive or compound symmetry may be used, with the final choice documented in the analysis report.

The primary confirmatory contrast will be the adjusted between-group difference in change from baseline to Week 20. Additional estimated contrasts at Week 4 and Week 8 will be reported as supportive longitudinal results. Model assumptions will be assessed through residual diagnostics. If strong deviations from normality or heteroscedasticity are detected, transformation or robust variance approaches may be used in supportive analyses, but the primary estimand and interpretation framework will remain unchanged.

10. Secondary efficacy analyses

10.1 Key secondary endpoints

The combined frailty index, cognitive composite, and metabolic/hepatic composite will each be analyzed using longitudinal mixed-effects models analogous to the primary model, adapted to the available visit schedule for each endpoint. If the primary endpoint does not achieve statistical significance, the key secondary analyses will be interpreted as supportive rather than confirmatory unless a predefined hierarchical testing sequence is successfully advanced.

10.2 Muscular secondary outcomes

- Continuous muscular outcomes (DEXA measures, GDLAM summary scores, ultrasound parameters, actigraphy-derived quantitative metrics, biomarkers, mitochondrial respiration metrics) will be analyzed with linear mixed-effects models or generalized linear mixed models as appropriate.
- Skewed biomarker distributions may be analyzed on the log scale; geometric mean ratios or back-transformed effects will be presented when relevant.
- The frailty phenotype category will be analyzed using ordinal logistic regression with repeated-measures extension when feasible; if proportional odds assumptions are not met, multinomial or binary category-collapsing approaches may be used as prespecified fallback strategies.

10.3 Cognitive and psychosocial outcomes

- The global cognitive composite is a key secondary endpoint and will be analyzed using mixed-effects models.
- Individual cognitive test scores will be analyzed as prespecified secondary outcomes using mixed-effects models, with transformation or non-Gaussian models when warranted by score distribution or ceiling/floor effects.
- Psychological well-being, subjective happiness, and life satisfaction will be analyzed using mixed-effects models on their original or transformed scales, depending on distributional properties.

10.4 Metabolic and hepatic outcomes

- Continuous fasting metabolic variables and liver enzyme outcomes will be analyzed using mixed-effects models.
- Binary or ordinal ultrasound-derived NAFLD outcomes will be analyzed using generalized estimating equations or mixed-effects logistic / ordinal logistic models, depending on the final data structure.
- Derived fibrosis scores will be analyzed as continuous endpoints when validated as such; otherwise categorical risk classifications will be analyzed using ordinal or binary models.

10.5 Safety analyses

Safety analyses will be descriptive and comparative. The number and proportion of participants with any adverse event, treatment-related adverse events, serious adverse events, and discontinuations due to adverse events will be summarized by group. Group comparisons may use Fisher exact tests, chi-square tests, or generalized linear models with robust standard errors when estimation of risk ratios or risk differences is preferable. Safety analyses will be performed in the safety population.

11. Multiplicity control

Because the trial includes multiple domains and repeated measurements, multiplicity will be handled explicitly. The primary endpoint will be tested at a two-sided alpha level of 0.05. Key secondary endpoints will be interpreted using a hierarchical gatekeeping approach in the following order: combined frailty index, global cognitive composite, and metabolic/hepatic composite. Formal confirmatory inference for a key secondary endpoint will proceed only if the preceding endpoint in the hierarchy is statistically significant at the allocated level.

All other secondary outcomes will be interpreted as supportive. Within families of related biomarkers or microbiome features, false discovery rate (FDR) control using the Benjamini-Hochberg procedure will be applied unless a more suitable domain-specific procedure is prespecified for the final laboratory platform.

12. Missing data

The primary repeated-measures analyses rely on likelihood-based mixed models, which provide valid inference under a missing-at-random assumption when the model is correctly specified. The amount, pattern, timing, and potential reasons for missingness will be described by treatment group. Sensitivity analyses using multiple imputation by chained equations may be conducted for major outcomes if missing data are non-trivial. Complete-case analyses will not be used as the sole basis for inference but may be shown as sensitivity checks.

13. Protocol deviations and sensitivity analyses

- A blinded protocol deviation review will classify major and minor deviations before database lock.
- A per-protocol analysis will be conducted for the primary endpoint as a sensitivity analysis.
- Additional sensitivity analyses may include alternative covariance structures, transformation of skewed outcomes, and models with additional clinically relevant baseline covariates.

14. Prespecified subgroup and effect-modification analyses

Baseline perceived stress is a prespecified effect modifier of interest. Rather than a purely post hoc subgroup analysis, the primary effect-modification analysis will incorporate baseline PSS-14 in interaction models. For the primary endpoint, a model including treatment, visit, baseline PSS-14, treatment-by-visit, treatment-by-PSS-14, visit-by-PSS-14, and treatment-by-visit-by-PSS-14 will be fit to evaluate whether the treatment effect differs according to baseline perceived stress.

A categorical supportive analysis may classify participants into lower versus medium/higher stress-anxiety groups using prespecified cut points or protocol-consistent definitions derived from PSS-14 and related scales. These subgroup analyses will be considered supportive because the trial is not powered primarily for interaction testing. Similar interaction analyses using baseline depression or anxiety scores may be performed as exploratory analyses.

15. Microbiome and mechanistic analyses

Microbiome analyses are prespecified as exploratory / mechanistic. Alpha-diversity metrics will be analyzed using mixed-effects models. Beta-diversity will be examined using appropriate distance-based approaches such as PERMANOVA with careful accounting for repeated measures where feasible. Differential abundance analyses of taxa or pathways will use methods suitable for sparse compositional microbiome data, such as ANCOM-BC, MaAsLin2, DESeq2, or other validated approaches chosen according to the final sequencing platform and preprocessing pipeline. The exact computational pipeline may depend on sequencing modality, but the inferential distinction remains fixed: these analyses are exploratory and intended to identify signatures associated with treatment response.

Associations between microbiome-derived features, short-chain fatty acids, and changes in muscular, cognitive, metabolic, or stress-related outcomes will be evaluated using correlation analyses and multivariable regression models. These association analyses are hypothesis-generating and will be clearly labeled as exploratory in any report or publication.

16. Derived variables and scoring rules

- The combined frailty index will be calculated according to the protocol-defined clinical and laboratory components.
- The global cognitive composite will be built from prespecified standardized scores after harmonizing direction so that higher values represent better cognition.
- The metabolic/hepatic composite will be built from prespecified standardized component measures after harmonizing direction so that higher values indicate better metabolic / hepatic status.
- Derived hepatic fibrosis scores and any binary or ordinal disease classifications will follow published formulae or protocol-defined cutoffs.

17. Interim analyses and blinding

No formal interim efficacy analysis is planned in this SAP unless explicitly mandated by an independent oversight body. Statistical programming, derivation of endpoints, and protocol deviation review should be finalized while treatment groups remain masked whenever feasible. Any unblinding prior to database lock will be documented and justified.

18. Tables, figures, and data presentation

- Participant flow will be summarized in a CONSORT-style diagram.
- Baseline characteristics will be displayed by randomized group.
- For longitudinal endpoints, plots of adjusted means with 95% confidence intervals over time will be presented.
- For microbiome analyses, diversity plots, ordinations, and differential-feature summaries will be clearly labeled as exploratory.
- All deviations from the SAP, if any, will be identified and justified in the final report.

19. Interpretation framework

The primary endpoint and the key secondary endpoints constitute the prespecified inferential backbone of this trial. Secondary and exploratory analyses may reveal additional associations or treatment-responsive domains that were not the focus of confirmatory testing. Such findings may be

biologically informative and may guide future research, but they will not supersede the prespecified interpretation of the primary and key secondary endpoints.

20. Signatures and document control

This document should be finalized, dated, archived, and, where applicable, publicly disclosed before database lock and before unblinded comparative analyses are undertaken. If uploaded to a trial registry or supplementary repository, the final file name should include the protocol or trial identifier, version number, and date.

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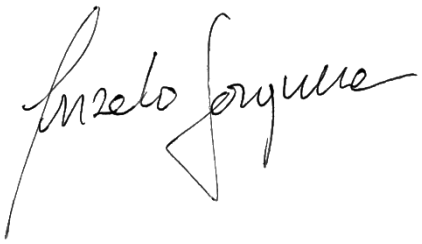
Principal Investigator	**Gonzalo Jorquera Olave, PhD** **CINUT & INTA-Universidad de Chile**	
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